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(54) Title: WOUND HEALING AGENTS

(57) Abstract

Platelet enriched plasma is produced from blood. The platelets are activated by thrombin which causes the release of platelet derived growth and angiogenesis factors. A carrier such as a microcrystalline collagen is added to produce a wound treating salve. The compound is applied directly to wounds and initiates healing in non-healing wounds as well as accelerating normal wound healing by increasing vascularization, stimulating fibroblast mitosis and migration and increasing collagen synthesis by fibroblasts.

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WOUND HEALING AGENTS

This application is a continuation-in-part of co-pending application Serial No. 676,471, filed November 29, 1984.

Field of the Invention

This invention relates to wound healing agents, specifically angiogenic and growth factors, their production from blood and their use to facilitate the healing of wounds.

Background of the Invention

Angiogenesis, which is the proliferation and directed growth of capillary endothelium, along with fibroplasia and collagen synthesis are integral components of a host's response to wounding. The activation of platelets and the clotting cascade are among the first reactions to injury.

Platelets activated by thrombin release a mitogen, or growth factor, for fibroblasts and smooth muscle cells and stimulate increased collagen synthesis by smooth muscle cells in vitro. The mitogen, (platelet-derived growth factor, hereinafter PDGF) is composed of two polypeptides. An article describing PDGF was published in 1982 by G.R. Grotendorst, T. Chang, H.E.J. Seppa, H.K. Kleinman and G.R. Martin in the Journal of Cellular Physiology entitled "Platelet-Derived Growth Factor is a Chemoattractant for Vascular Smooth Muscle Cells", Vol. 113, pp. 261-266. The article is incorporated herein by reference.

A non-mitogenic substance, called angiogenic factor, is also produced by thrombin activated platelets and stimulates capillary growth. Various angiogenesis factors are known including tumor, retinal and wound fluid angiogenesis factors. It is unknown whether all angiogenesis factors share a common mechanism of action upon capillary endothelial cells.

Angiogenesis factors were isolated and described

by M.S. Banda, D.R. Knighton, T.K. Hunt and Z. Werb in Proc. Nat'l. Acad. Sci. U.S.A. (7773 - 7777, Dec. 1982), in an article entitled "Isolation of a nonmitogenic angiogenesis factor from wound fluid", the disclosure of which is incorporated herein by reference.

Angiogenesis and platelet derived growth factors are described by D.R. Knighton, T.K. Hunt, K.K. Thakral and W.H. Goodson III, in "Role of Platelets and Fibrin in the Healing Sequence," Annals of Surgery 196: 379-388 (1982), the disclosure of which is incorporated by reference. In this article, the successful treatment of a non-healing wound in a patient is described in which a single, ten-unit platelet transfusion was given. The wound healed in three weeks.

A recent study has indicated that when the body's normal healing process works, it is only at about a 50% effectiveness level.

A human angiogenic factor is produced from human foreskin fibroblasts in United States Patent 4,273,871 to Tolbert et al. A publically available foreskin fibroblast cell line is utilized to produce an angiogenic factor.

In United States Patent 4,479,896 to Antoniades the disclosure of which is incorporated herein by reference, platelet-derived growth factors are characterized and extracted for study by gel electrophoresis means.

Brief Summary of the Invention

Thrombin activated platelets have the capacity to stimulate angiogenesis, increased collagen synthesis and cell division and growth. It has been found that samples of whole blood may be utilized to prepare a platelet-enriched plasma, which when activated by thrombin, contains angiogenic and growth factors which may be used to speed the healing process of wounds.

Blood is stabilized and centrifuged to obtain a

platelet-rich plasma. The blood is stabilized by mixing with citrate-phosphate-dextrose in a ratio of 1:5 (20% solution). The platelet-rich plasma (hereinafter PRP) is preferably centrifuged again until a high concentration of platelets is obtained. The platelets are then placed in a platelet buffer. The concentration of platelets should be at least 1,000,000 platelets per milliliter. Preferably, the concentration should be on the order of 1,000,000,000 platelets per milliliter.

Thrombin is added to the PRP in order to activate the platelets. Preferably, about 1 to about 10 units of thrombin are utilized per milliliter of PRP. The thrombin-activated platelets release platelet derived growth factors (hereinafter PDGF) and platelet derived angiogenesis factors (hereinafter PDAF). The platlets and thrombin are allowed to incubate at room temperature for about 5 to 10 minutes.

The activated PRP containing PDGF and PDAF is preferably added to a biologically compatible macromolecular substance which acts as a carrier. First the platlets are centrifuged at about 950 x g and the platelet free supernatant is mixed with the carrier. Preferably, a microcrystalline collagen such as Avitene® brand collagen as sold by FMC Corp., Avicel Dept., Marcus Hook, PA 19061 is utilized as the biologically compatible carrier. Microcrystalline collagens are biologically compatible in the body. Enough carrier is added to soak up all the platelet rich plasma that is obtained from the blood. For example, a 40ml blood sample would typically require about 25ml of carrier after enrichment. The paste so obtained is preferably stored on ice or in the refrigerator.

The pharmaceutical preparations for use as a wound dressing sold by Pharmacia Fine Chemicals, Inc. of Piscataway, New Jersey under the trademark Debrisan is a suitable carrier.

The activated PRP within the carrier may then be applied to a wound. The highly enriched and active PDGF and PDAF therewithin assists in healing by proliferating and directing the growth of capillary endothelium, doubling the rate of collagen synthesis and by producing leukocyte chemotaxis. Mitogenic activity results in cellular division and growth to replace the lost tissue.

Daily application of the activated PRP to wounds stimulates and bolsters the healing sequence. The amount of PRP processed from 40ml of blood is enough to produce applications for seven days. The material is placed over the entire wound at a relatively uniform thickness, approximately two millimeters thick. Granulation, contraction and epithelization may be initiated through the use of activated PRP where the body's own repair signals are inadequate to stimulate good healing.

Whenever thrombin is used herein, it is referring to thrombin as a biologic release agent for platelet release. Other biologic release agents known in the art, including collagen, ADP and serotonin, may be utilized instead of or in addition to thrombin to activate the platelets, although thrombin is preferred.

Detailed Description of the Invention

Blood obtained from the individual to be treated with the wound healing factors of the invention is stabilized in siliconized tubes containing acid-citrate dextrose (0.15M citrate, 2% glucose, pH 4.2) (hereinafter CPD) and is centrifuged in order to separate out the platelet-rich plasma therefrom. Forty to sixth milliliters of blood combined with 4-6ml of CPD is then centrifuged at about 135 x g for 20 minutes at about 4°C to obtain platelet-rich plasma. The platelet rich plasma is removed and placed into another sterile, 50ml tube. A platelet count is then taken. The CDP is utilized to prevent activation of the clotting sequence by contact of the blood

with the plastic in the syringe. The CPD is present in the syringe while the blood is withdrawn from the patient. The blood is continuously mixed with the CPD to prevent coagulation. The platelet-rich plasma in the tube is then centrifuged at 750 x g for 10 minutes at 4°C.

The platelet-free plasma is removed and discarded. The platelet pellet is resuspended in a quantity of platelet buffer to produce a final ml. A lower concentration of about a million platelets per ml is useful, but is less preferred. The platelet buffer utilized contains .05 M HEPES (N-2-hydroxyethylpiperazine-n-2-ethanesulfonic acid), 0.03 M glucose, 0.004 M KCl, 0.1 M NaCl and about 0.35% human serum albumin adjusted to a pH of about 6.5. A sample is frozen at about -20°C for later testing of mitogenic activity. Another sample is streaked onto blood agar as a sterility test.

The platelet-rich plasma is the only blood fraction utilized in the processes and compositions of the invention. The PRP is then activated with purified thrombin at a rate of about 1 to about 10 units of thrombin per milliliter of PRP. Preferably, about 1 unit of thrombin per ml of platelet-rich plasma is utilized. The activity of the thrombin coagulates the fibrinogen and activates platelets causing them to release alpha granules containing platelet-derived growth factor and platelet-derived angiogenesis factor. The thrombin used was Thrombinar™ brand from Armour Pharmaceutical Co. of Kankakee, Illinois. The platetlets and thrombin are allowed to incubate at room temperature for about 5-10 minutes.

The PRP is then subjected to a removal of platelets and fibrin by centrifugation. The resulting supernatant contains both PDAF and PDGF after centrifuging at 950 x g for about 5 minutes at 4°C. The pellet is discarded since the PDAF & PDGF have been extracted into the supernatant. PDGF has been isolated and characterized.

It is a protein of 30,000 molecular weight which breaks down into two molecular weight species of 15,000 and 14,000 molecular weight.

In order to apply the PDAF and PDGF in the platelet-free supernatant thus obtained to a wound, it is desirable to utilize a carrier substance which is biologically compatible and acts as a temporary "depot". A macromolecular substance such as microcrystalline collagen provides a suitable carrier. An especially preferred carrier is Avitene® brand microcrystalline collagen from FMC Corp., Avicel Dept., Marcus Hook, PA 19061. The resultant composition is thicker and will tend to remain in position in contact with the wound. Debrisan™ brand wound dressing which contains Sepharose™ brand beads, trademarks of Pharmacia Fine Chemicals, Inc. of Piscataway, New Jersey, may be utilized as an alternative carrier. Preferably, about 8-10ml of supernatant per gram of carrier is used to produce a paste.

Application of the wound treating composition is by physically applying the material over an into the wound as in applying a medicated salve. Treatments should be repeated on a daily basis as long as the wound remains open. A preferred treatment is to apply an approximately one mm thick dressing of the platelet factor/carrier complex to the wound in the morning. It is then dressed with a sterile, dry dressing. In the evening, the dressing is removed and the substance is removed by washing with sterile saline.

Although the clinical testing involving the wound treating compositions of the invention have been directed to wounds on the body exterior, the compositions may treat internal wounds as well. Sutures may be impregnated with the wound treating compositions to speed internal healing. The wound treating compositions may also be used in conjunction with biodegradable dressings, as a coating over

implantable devices and biodegradable devices utilized in surgical procedures. Generally, any foreign body to be inserted into a patient may be coated with the composition to speed the healing process. Alternatively, the composition may be applied over the damaged tissue directly.

Initial clinical trials have been performed on eight patients, all with nonhealing wounds from periods of one to five years. All patients had maximal standardized care in attempts to heal the wounds. That therapy had failed. In all cases, administration of platelet-derived factors initiated a healing response as evidenced by granulation tissue formation (granulation tissue contains fibroblasts, endothelial cells and collagen). The wounds closed by contraction and epithelialization or by skin grafting. Stimulation of healing and eventual repair occurred in all applications.

While it is preferred to prepare activated PRP for wound treatment purposes directly from the injured animal's own blood, the advantages of the invention may be achieved by using blood or outdated platelets from animals of the same species. Utilization of blood from the injured individual to be treated is especially preferred since it avoids exposure to possible hepatitis or other contaminants from banked blood. The use of a patient's own blood would also eliminate any possible allergic reactions. A consistent source of the material may be obtained from washed, outdated human platelets. The substances may also be utilized in veterinary applications by utilizing platelets derived from the animal itself or another animal within the same species.

Example I

A patient having an open wound on the left foot following debridement of dead tissue and transmetatarsal amputation was started on PDGF and PDAF obtained as described above from his own blood. After the treatment

protocol, the wound was filled with new granulation tissue. A subsequent debridement showed completely covered metatarsal bones and contracture of the sizable wound.

Example II

A patient underwent amputation of his right great toe and was treated with standard therapy for three weeks without any granulation tissue accumulating within the wound. He was then started on the platelet factor therapy of the invention. After three weeks of treatment, the wound contracted approximately 30-40% and was healing rapidly.

Example III

A patient having two large wounds on the medial and lateral aspect of his transmatatarsal amputation stump had been treated for four months without healing using conventional therapy. Within two weeks of treatment with PDAF and PDGF as described above, the wound had cleared of an apparent infection and started producing granulation tissue.

Thirty-eight nonhealing ulcers from 28 diabetic patients were treated with the PRP paste. The average duration of the ulcers before treatment was 6-1/2 years. A paste prepared from PRP at a concentration of about 10^9 platelets/ml was combined with Avitene brand collagen. The patients applied the PDGF and PDAF containing paste daily for 12 hour periods for an average of 8 weeks. Each day, the wounds were debrided of dead tissue. All of the wounds produced granulation tissue and closed an average of 83% when compared to starting wound area. Ninety-five percent of the ulcers were successfully treated resulting in either total wound epithlialization or successful skin grafting. Only two of these nonhealing wounds did not heal. The healed ulcers remain closed with no evidence of hypertrophic scar formation or neoplastic formation.

In considering this invention, it should be

remembered that the disclosure is illustrative only, and that the scope of the invention should be determined by the appended claims.

WHAT IS CLAIMED IS:

1. In a process for producing physiologically active wound healing substances, the steps comprising:

a) mixing blood with a citrate phosphate dextrose solution;

b) isolating platelet-rich plasma from said blood;

c) activating said platelets; and

d) combining said activated platelet-rich plasma with a microcrystalline collagen carrier.

2. The process of claim 1 in which the platelets are activated with thrombin.

3. A process for the production of platelet-derived growth and angiogenesis factors which comprises:

a) obtaining a platelet-rich plasma solution from blood; and

b) activating said platelets so as to produce platelet-derived growth and angiogenesis factors.

4. The process of claim 3 wherein the platelets are activated with thrombin.

5. A process for producing wound healing substances comprising:

a) obtaining a sample of blood from an animal;

b) isolating a platelet-rich plasma from said blood sample;

c) activating said platelets with about 1-10U thrombin per milliliter of platelet-rich plasma.

6. A process for the production of platelet-derived angiogenic and growth factors in vitro comprising:

a) obtaining a sample of blood;

b) isolating a platelet-enriched plasma fraction from said blood;

c) activating the platelets with thrombin; and

d) isolating the resulting angiogenic and growth factors from said activated fraction.

7. A composition for the treatment of wounds comprising:

platelet-derived angiogenic and growth factors and a carrier.

8. The composition of claim 7 wherein said carrier is a microcrystalline collagen.

9. A method for enhancing wound repair comprising:

a) coating a wound with the composition of claim 7.

10. A method for treating wounds which comprises administering the composition of claim 7 to the wound daily.

11. A process for extracting from blood a platelet derived angiogenesis factor and growth factor comprising the steps of:

a) providing a volume of blood;

b) adding an anticoagulent to said blood, said anticoagulant being of the type which may be used for preventing coagulation within a body;

c) isolating a platelet enriched plasma fraction from said blood, said platelets being in a concentration of at least 1,000,000 per milliliter of blood, and

d) adding from about 1 to about 10 units of thrombin per 10 milliliters of said platelet enriched plasma.

12. The process of claim 11 wherein said platelets are concentrated to a concentration of about 1,000,000,000 per milliliter.

13. A process for producing wound healing substances comprising:

a) obtaining a sample of platelets;

b) isolating a platelet-rich plasma from outdated platelets;

c) washing said platelets to remove serum containing material;

d) activating said platelets with from about 1 to about 10 units of thrombin per milliliter of platelet-rich plasma.

14. The process of claim 13 wherein about 1 unit of thrombin is utilized per milliliter of platelet-rich plasma.

15. The process of claim 13 wherein said platelets are obtained from outdated platelets in a blood bank.

16. A composition for the treatment of wounds comprising:

a) a platelet-derived angiogenesis and growth factors in a platelet-rich plasma having a concentration of at least about 1,000,000,000 platelets per milliliter, and

b) about 1 to about 10 units of thrombin per milliliter of platelet-rich plasma.

17. The composition of claim 16 further including a pharmaceutically acceptable carrier.

18. The composition of claim 17 wherein said carrier is a microcrystalline collagen.

19. The composition of claim 16 wherein the platelet concentration is about 1,000,000,000 per milliliter.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/02205

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. 424/101 IPC[4] A61K 35/14

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	424/101 514/2, 773 & 774

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CHEMICAL ABSTRACTS 10TH COLLECTIVE TO DATE VOL. 86-101 1977-1985
"BLOOD-PLATELET" "COLLAGEN" "ANIMAL GROWTH FACTOR"

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,479,896 Published Oct. 30, 1984 Antoniades	1-19
Y	US, A, 3,628,974 Published 21 December 1971 Battista	1-19
Y	N, Annals of Surgery Vol. 196 No. 4 (Oct. 1982) Knighton et al, Role of Platelets and Fibrin in the Healing Sequence, pages 379-388	1-19

* Special categories of cited documents: [15]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
23 January 1986	31 JAN 1986
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	SAM ROSEN

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